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Occurrence, characterization and development of two different types of microbodies in the nematophagous fungus *Arthrobotrys oligospora*

(Biogenesis of microbodies; cytochemistry; catalase; nematophagous fungus)

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1. SUMMARY

The occurrence of microbodies in different cells of the nematophagous fungus *Arthrobotrys oligospora* has been investigated. In the predacious phase this organism forms complex 3-dimensional network traps. Mature trap cells generally were crowded with "special" microbodies which possessed an electron dense matrix and were surrounded by a membrane of approx. 9 nm. These organelles developed during the early stages of trap formation and were derived from specialized regions of the endoplasmic reticulum. Cytochemical staining experiments revealed that the electron-dense microbodies contained catalase and D-amino acid oxidase and thus must be considered peroxisomal in nature. Electron-dense bodies were absent in normal vegetative cells of the fungus. These cells contained "normal" microbodies which developed from each other by the separation of small organelles from mature ones. As in yeasts, the metabolic function of these latter organelles was dependent upon environmental conditions.

2. INTRODUCTION

Microbodies, first recognized as separate cell constituents in 1954 [1], are currently considered to be ubiquitously present in eukaryotic cells. These organelles are generally characterized by a simple morphology, but show a great diversity in enzyme repertoires and, as a consequence, may be involved in a variety of metabolic processes [2]. For instance, in plants they may participate in seed germination, nitrogen metabolism and photorespiration [3–5], while in fungi they may be involved in carbon and/or nitrogen metabolism and sporulation or germination processes [6–8].

Recently, a morphologically different type of microbodies was detected in cells of the nematode-trapping structures of the fungus *A. oligospora*. This fungus can be readily transformed from a saprophytic phase, consisting of normal hyphae, to a predacious phase with characteristic three-dimensional adhesive network traps involved in nematode capturing [9]. Both hyphae and traps are vegetative mycelial structures, but they differ greatly, both structurally and functionally [10]. The individual cells of the traps contain large

numbers of electron-dense bodies, which are absent in normal hyphal cells [10–12].

In this paper evidence is presented that the microbodies occurring in the trap cells of *A. oligospora* are peroxisomal in nature. They develop from specialized regions of the endoplasmic reticulum by a unique process that is entirely different from that involved in the development of microbodies present in normal hyphal cells of the fungus.

3. MATERIALS AND METHODS

3.1. Organisms and cultivation methods

A. oligospora Fres. (ATCC24927) was grown on dialysis membranes placed on the surface of a low nutrient medium [13] that was supplemented with a trap-inducing peptide as described previously [9]. For comparison the organism was also grown as hyphae without traps in 500-ml shake flasks containing 200 ml of the mineral medium of Van Dijken et al. [14]. To this medium was added glucose (0.25% w/v) or ethanol (0.25% v/v) as the carbon source and ammonium sulphate (0.25% w/v) or methylamine (0.2% w/v) as a nitrogen source. In capturing experiments the bacteria-feeding nematode *Panagrellus redivivus* was used [11].

3.2. Biochemical experiments

Cell-free extracts were prepared by sonication of whole cells [14]. D-Amino acid oxidase and amine oxidase were assayed with an oxygen electrode [14] and catalase by the method of Lück [15]. Protein was measured according to Lowry et al. [16], using bovine serum albumin as the standard.

3.3. Freeze-etching

Fungal hyphae were incubated in 10% (v/v) glycerol for 2–5 min, frozen in freon and freeze-fractured in a Balzer's freeze-etch unit according to Moor [17].

3.4. Electron microscopy

Hyphae from liquid cultures were harvested by centrifugation. For kinetic studies on trap development and capturing and/or digestion of nematodes the fungus was grown on dialysis membranes [12,13]. Fixation and post-fixation after cytochemical experiments, cytochemical staining of catalase, D-amino acid oxidase, amine oxidase, acid phosphatase and glucose-6-phosphatase were performed as described previously [18,19].

4. RESULTS

4.1. Biochemistry

Cell-free extracts of hyphae of *A. oligospora* grown in shake flask cultures on glucose or ethanol as a C-source and ammonium sulphate as the N-source contained low levels of catalase activity. In addition to catalase, glucose-grown cells contained only a low activity of D-amino acid oxidase, but several other known microbody-matrix enzymes [7] were undetectable under these conditions. As expected in ethanol-grown cells enhanced levels of isocitrate lyase and malate synthase were observed (not shown), whereas amine oxidase was present in cultures containing methylamine instead

Table 1

Activities of different microbody-matrix enzymes in cell-free extracts of *Arthrobotrys oligospora*, grown for 36 h in shake flask cultures on various media or at different stages of trap development during growth on dialysis membranes on an agar surface on a low-nutrient medium in the presence of a trap-inducing peptide

Growth conditions	Enzyme activities		
	Catalase	D-amino acid oxidase	Amine oxidase
Batch cultures			
Glucose/$(NH_4)_2SO_4$	4	6	–
Glucose/CH_3NH_2	9	–	41
Ethanol/$(NH_4)_2SO_4$	13	–	–
Ethanol/CH_3NH_2	10	–	28
Agar cultures			
Without traps	2	–	–
Agar cultures			
With traps			
7 days old	44	39	–
20 days old	66	65	–

Catalase is expressed as $\Delta E_{240} \cdot min^{-1} \cdot mg\ protein^{-1}$, oxidase activities as nmol $O_2 \cdot min^{-1} \cdot mg\ protein^{-1}$.
–, no activity detected.

of ammonium sulphate as the N-source (Table 1). When *A. oligospora* was grown on the surface of a low nutrient agar medium [9,10], only very low activities of catalase and D-amino acid oxidase were detected prior to trap formation. However, during trap development, an increase in activities of both enzymes was observed (Table 1). This increase in enzyme activities was clearly associated with the process of trap formation since a further increase was observed during the subsequent maturation of the traps (Table 1).

4.2. Electron microscopy

4.2.1. Normal hyphal cells

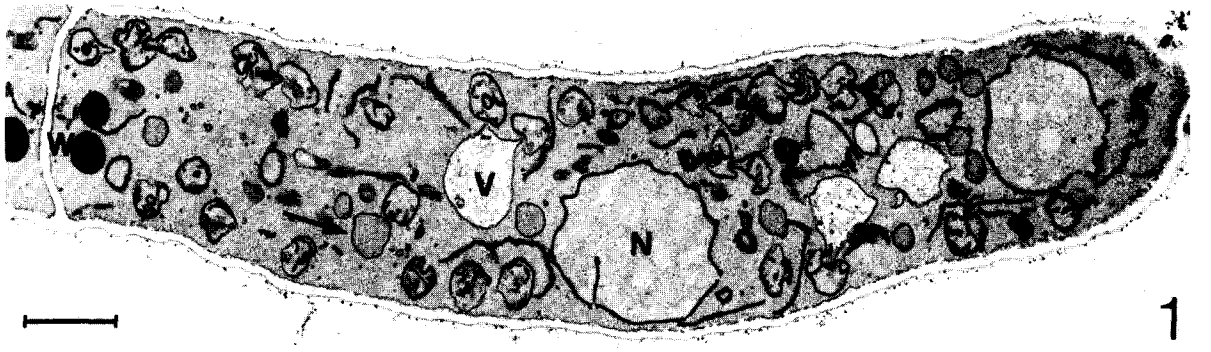
Individual hyphal cells present in a culture of *A. oligospora* grown for 36 h in shake flasks on glucose/ammonium sulphate medium contained, besides the usual cell organelles as nuclei, mitochondria and vacuoles, a limited number of small microbodies which were scattered throughout the cytoplasm. In thin sections of $KMnO_4$-fixed cells these organelles were irregular in shape with dimensions up to 0.2 μm. Transfer of such cells into media which contained methylamine instead of ammonium sulphate as a nitrogen source, resulted in a significant increase in both size and number of the microbodies. Observations made during the initial hours after transfer indicated that these large microbodies originate from the small organelles originally present in the glucose/ammonium sulphate-grown cells, in a fashion similar to that described for yeast microbodies [7,8]. In longitudinal sections of cells cultivated in glucose/methylamine media generally 8 to 12 microbodies were observed which were irregular in shape and with dimensions up to 0.5 μm. A similar increase in microbody-size and number was observed after transfer of glucose/ammonium sulphate-grown cells into ethanol/ammoniumsulphate- or ethanol/methylamine-containing media. After 36 h of cultivation in thin sections of these cells generally 10 to 15 microbodies were observed, irregular in shape with dimensions up to 0.5 μm (Fig. 1). During growth on ethanol the organelles were generally in close proximity to the mitochondria.

Microbodies were also frequently observed in normal hyphal cells that developed on the surface of a low nutrient agar medium in the presence of a trap-inducing peptide prior to the formation of traps. As described above for cells grown in liquid cultures, the organelles were randomly distributed over the cells; they were irregularly shaped and measured up to 0.6 μm (Fig. 2).

Irrespective of the growth conditions, the microbodies present in hyphal cells of *A. oligospora* had a number of properties in common. They frequently existed in close association with strands of endoplasmic reticulum (cf. Figs. 1 and 2) and lacked crystalline inclusions, as was also evident in glutaraldehyde-OsO_4/$K_2Cr_2O_7$-fixed cells. In addition, their surrounding membranes, approx. 70 Å in width in thin sections, showed smooth fracture faces in freeze-etch replicas, a property that is shared with the membrane of yeast microbodies [7,8].

4.2.2. Trap cells

Individual cells of mature traps of *A. oligospora* are characterized by the presence of large numbers of microbodies [10,12]. These organelles, which are completely lacking in normal hyphal cells from either liquid or solid media, are largely uniform in size in mature trap cells and measure approx. 0.35 μm. They show an electron-dense matrix, both after glutaraldehyde-OsO_4/$K_2Cr_2O_7$- and $KMnO_4$-fixation, lack crystalline inclusions and are surrounded by a single membrane of approx. 9 nm. The origin and development of these microbodies was studied in a number of kinetic experiments using the recently developed dialysis membrane technique [12] which enables sectioning of individual traps at different stages of development for electron microscopical observations. Our results indicate that the development of the typical electron dense microbodies started at the initial stages of trap formation. Developing trap cells show a high metabolic activity as judged by the abundance of different cell components as mitochondria, endoplasmic reticulum and small vesicles. During the initial stages of their development — judged by light microscopy — the cells already contained a relatively high number of microbodies of different sizes (Fig. 3). Observations of $KMnO_4$-fixed cells revealed that the electron-



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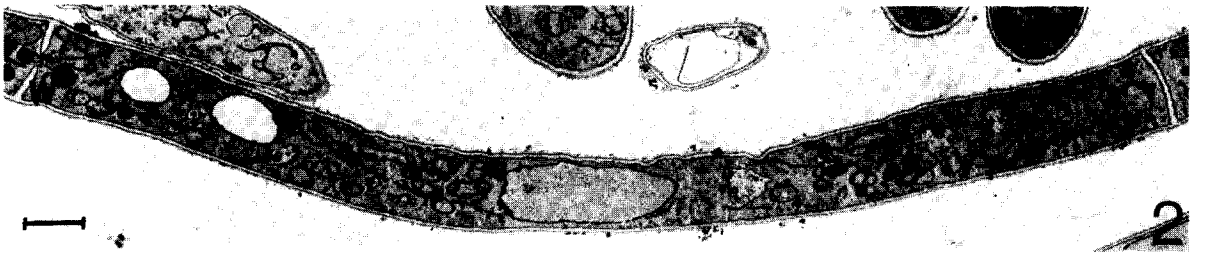

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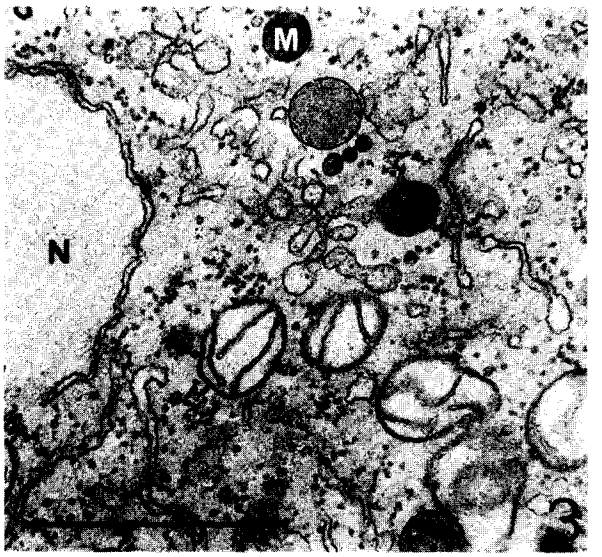

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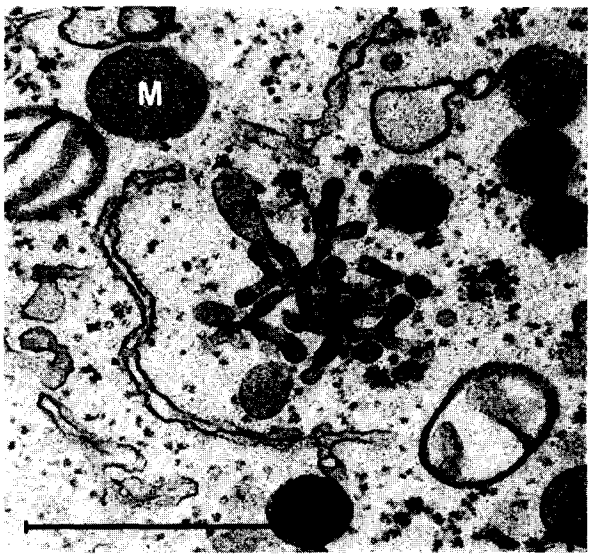

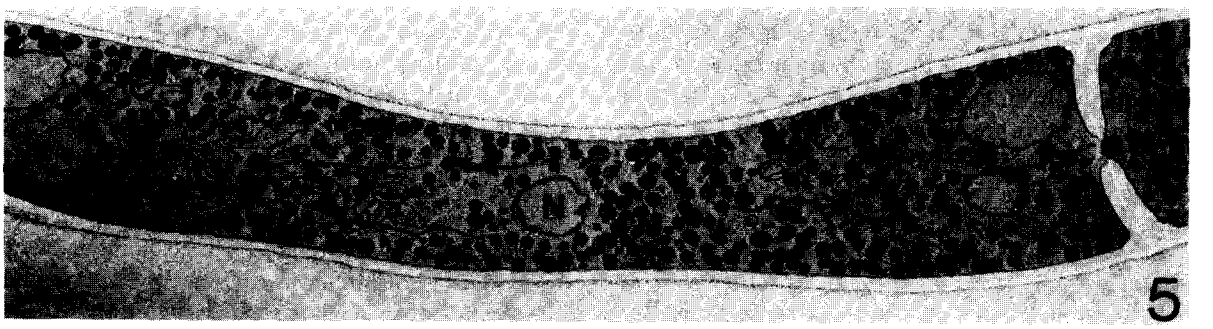

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dense microbodies originated from specialized regions of endoplasmic reticulum (Fig. 4) by budding and subsequently increased in size. Serial sectioning revealed that the membranous regions may vary from simple structures, consisting of 2 or 3 tubuli, running in different directions up to the complex 3-dimensional tubular network as depicted in Fig. 4. Development of the electron-dense microbodies is most probably not associated with the presence of the small vesicles in the cells, since direct associations or fusions of these vesicles with the developing electron-dense microbodies were never observed. In young trap cells the development of these microbodies was not confined to a single specialized region of endoplasmic reticulum; serial sections revealed that up to four reticular regions may be active in one cell, which may explain the observed rapid increase in the number of microbodies per cell. Individual mature trap cells are generally crowded with these organelles, whereas the number of mitochondria and the amount of endoplasmic reticulum had considerably decreased (Fig. 5). Mature trap cells also lacked the small vesicles, characteristically present in young cells (cf. Figs. 3 and 5).

The electron-dense microbodies, described above, have also been detected in nematode-penetrating hyphae. Penetration of nematodes generally occurred within 1–2 h after capturing [9,12]. Upon invasion, the first cell of the penetrating hyphae generally was bulb-shaped and contained many electron-dense microbodies (Fig. 6).

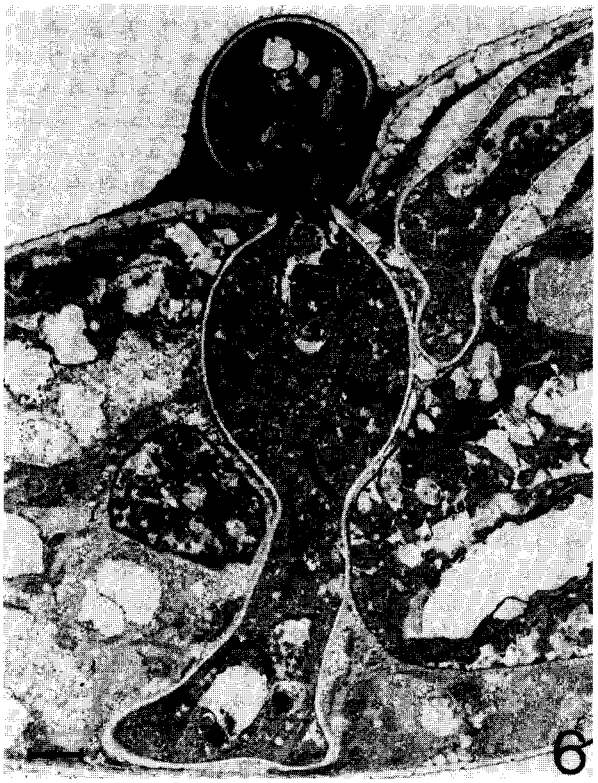
4.3. Cytochemistry

The subcellular location of catalase activity in *A. oligospora* was investigated with the conventional DAB-method and involved incubation of glutaraldehyde-fixed cells with DAB and exogenous H_2O_2 [18]. Incubations of hyphal cells, grown in shake-flask cultures in media containing glucose or ethanol as the C-source and ammonium sulphate or methylamine as the N-source all yielded positively stained microbodies (Fig. 7). Similar results were obtained with trap cells, grown on a low nutrient medium. The electron-dense microbodies, present in these cells all were intensely stained after incubations with DAB and H_2O_2 (Fig. 8). Control experiments, performed in the presence of aminotriazole to inhibit catalase activity or with DAB in the absence of H_2O_2 showed unstained microbodies. In all incubations with DAB also the mitochondria were intensively stained. However, this staining was independent of the presence of H_2O_2, was prevented by adding cyanide to the incubation mixtures and therefore was attributed to activity of mitochondrial peroxidases [18,20]. Oxidase activities were demonstrated with the cerium method. This involved incubation of glutaraldehyde-fixed cells with $CeCl_3$ in the presence of the oxidase-specific substrate under aerobic conditions [18]. Incubation of hyphal cells, grown on methylamine as the N-source with $CeCl_3$ and methylamine resulted in positively stained microbodies, indicating the presence of amine oxidase activity in these organelles (Fig. 9). In addition to catalase, the microbodies in trap cells of *A. oligospora* contained D-amino acid oxidase as was demonstrated after incubations with $CeCl_3$ and D-alanine (Fig. 10). Incubations in the presence of urate or glycollate yielded negative results, indicating the absence of urate oxidase and L-α-hydroxy acid oxidase, respectively.

Previously it has been suggested that the trap-bound microbodies might carry hydrolytic enzymes and thus be involved in nematode penetra-

Micrographs are taken from cells of *A. oligospora*, (post)fixed with $KMnO_4$. The bar represents 1 μm. Abbreviations: M, microbody; N, nucleus; V, vacuole; W, Woronin body.

Figs. 1, 2. Survey of cells of normal hyphae, grown in batch cultures on ethanol/ammoniumsulphate (Fig. 1) or on a dialysis membrane on a low nutrient agar medium (Fig. 2). In the cells many microbody profiles can be observed (arrows). In the vicinity of the cross-wall Woronin bodies are present. **Figs. 3 and 4.** Details of trap cells in an early developmental stage showing the presence of electron-dense microbodies of different sizes together with numerous small vesicles (Fig. 3). The electron-dense microbodies developed from specialized regions of the endoplasmic reticulum (Fig. 4). **Fig. 5.** Survey of a mature trap cell, crowded with electron-dense microbodies. **Fig. 6.** Survey of the area of penetration of an infected nematode 6 h after capturing, showing the typical bulb-shaped penetration hypha and the presence of electron-dense microbodies in this hypha.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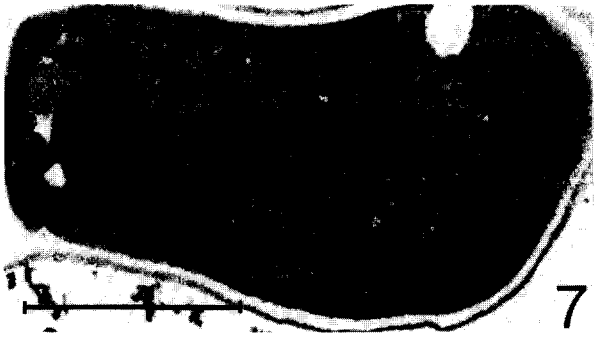
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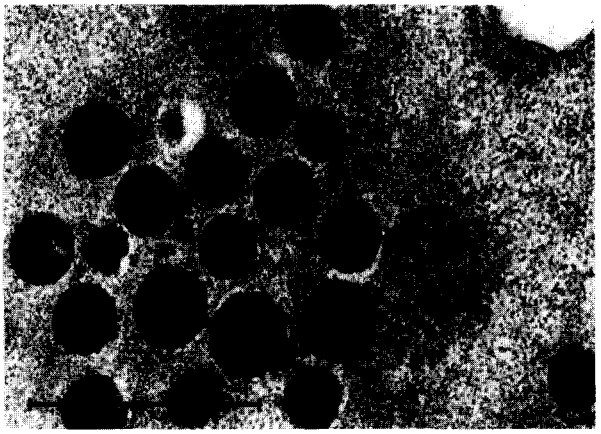

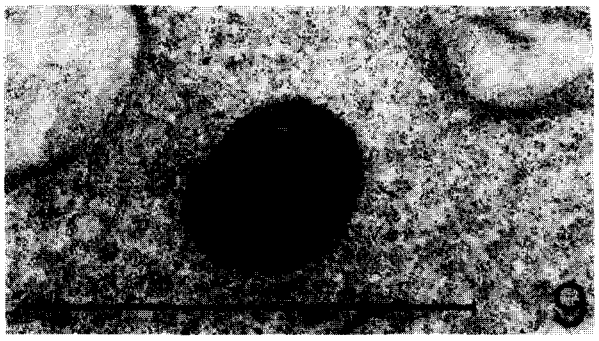
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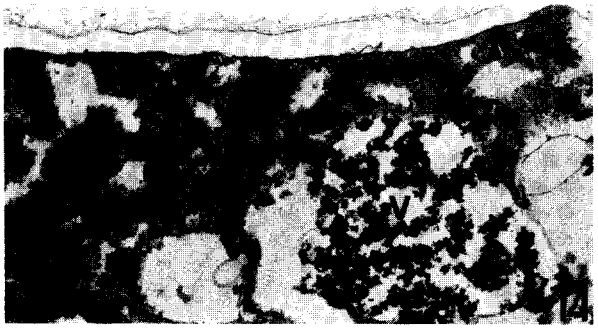
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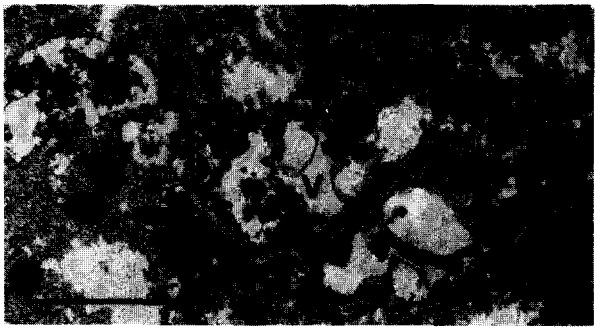
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tion and/or digestion [11]. For this reason experiments were performed to localize acid phosphatase and glucose-6-phosphatase, key enzymes of fungal hydrolytic compartments [19] in these trap cells. Incubations of glutaraldehyde-fixed cells with $CeCl_3$ and β-glycerophosphate or glucose-6-phosphate, respectively [19], revealed that enzyme-specific reaction products were solely present in the vacuoles (Figs. 11, 12). The other cell components, including the microbodies, invariably remained unstained indicating that the activity of these hydrolytic enzymes was confined to the vacuoles.

5. DISCUSSION

Microbodies have been shown to occur in all eukaryotic cells studied so far, including filamentous fungi and yeasts [6,7,21]. Proliferation of microbodies in the latter organisms is largely prescribed by environmental conditions; depending on the composition of the cultivation media organelles develop which may be involved in carbon and/or nitrogen metabolism [7,8,22] while, in addition, they also may harbour biosynthetic functions [23]. The biogenesis of yeast microbodies deviates from the classical model proposed by de Duve and Baudhuin [24] which involves development of the organelles as outgrowths of smooth endoplasmic reticulum. The evidence obtained so far indicates that in yeast microbodies develop from already existing organelles [7,8,25]. The present results, obtained with *A. oligospora* strongly suggest that identical mechanisms may operate in the development of microbodies in hyphal cells of filamentous fungi. This is particularly indicated by the results of the different transfer experiments. For instance, the peroxisomes present in glucose/methylamine-grown hyphae developed from those originally present in the inoculum cells (grown on glucose/ammonium sulphate) by the import of amine oxidase protein. Similarly, the organelles developed into glyoxysomes after transfer of hyphae into ethanol/ammonium sulphate-containing media. Furthermore, since all the individual microbodies present in ethanol/methylamine grown hyphae contained amine oxidase activity, also intermediate forms which harbour both glyoxysomal and peroxisomal functions may be present.

Thus, during growth of normal hyphae of *A. oligospora*, the development and subsequent multiplication of microbodies is most probably identical to the mechanisms described for yeasts in that these organelles develop from already existing ones. On the other hand, a completely different situation was encountered in cells from the predacious phase of this fungus. Our combined ultrastructural and biochemical results clearly indicate that the electron-dense bodies which develop in trap cells of *A. oligospora*, contain catalase and D-amino acid oxidase and therefore are peroxisomal in nature. However, apart from the unprecedented number in which they are present, the individual organelles present in trap cells differ in morphology from those present in hyphae in two respects. First, they are surrounded by a relatively thick membrane of approx. 9 nm which does not reveal smooth fracture faces in freeze-etch preparations (M. Veenhuis, unpublished observations); second, the microbody matrix shows enhanced electron density compared to the matrix of 'normal' microbodies. This latter property may indicate the presence of additional matrix proteins which are yet unknown. This may extend the physiological function of the organelles beyond that of being solely peroxisomal.

Apart from the morphological differences mentioned above, a major difference concerns the biogenesis of the organelles. In trap cells they developed as outgrowths of specialized regions of endoplasmic reticulum; development from already ex-

Figs. 7–10. Cytochemical demonstration of catalase (Fig. 7) and amine oxidase (Fig. 9) in microbodies in cells of normal hyphae grown on glucose/methylamine. In the matrix of the electron dense microbodies of mature trap cells catalase (Fig. 8) and D-amino acid oxidase (Fig. 10) can be demonstrated. Catalase was stained with DAB and H_2O_2, the oxidases with $CeCl_3$ in the presence of enzyme-specific substrates. **Figs. 11, 12.** Cytochemical demonstration of acid phosphatase (Fig. 11) and glucose-6-phosphatase (Fig. 12) in mature trap cells. Enzyme-specific reaction products are confined to the vacuoles. Incubations: $CeCl_3$ in the presence of β-glycerophosphate or glucose-6-phosphate, respectively.

isting microbodies by the separation of small organelles from mature ones [7,8], as was evident in hyphal cells, was not observed. This mode of microbody synthesis is unique because it has never been observed previously in any eukaryotic cell. It provides the first example of the involvement of endoplasmic reticulum in this process. Although the process of microbody formation in trap cells reported here cannot be explained on the basis of the known models for microbody biogenesis, it does unify elements of the classical model of de Duve and Baudhuin [24] and that of the model postulated recently by Lazarow et al. [26], which involves development of new microbodies from a microbody reticulum.

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